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Research Report/ Technical Spotlight / New Method

Everyday memory: towards a translationally effective method of modeling the encoding, forgetting and enhancement of memory.

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ABSTRACT

The testing of cognitive enhancers could benefit from the development of novel behavioural tasks that display better translational relevance for daily memory, and permit the examination of potential targets in a within-subjects manner with less variability. We here outline an optimized spatial ‘everyday memory’ task. We calibrate it systematically by interrogating certain well-established determinants of memory, and consider its potential for revealing novel features of encoding-related gene activation. Rats were trained in an event arena in which food was hidden in sandwells in a different location everyday. They found the food during an initial memory-encoding trial and were then required to remember the location in 6-alternative choice- or probe trials at various time-points later. Training continued daily over a period of 4 months, realising a stable high level of performance and characterised by delay-dependent forgetting over 24 h. Spaced but not massed access to multiple rewards enhanced the persistence of memory, as did post-encoding administration of the PDE4 inhibitor Rolipram. Quantitative PCR and then genome wide-analysis of gene-expression led to a new observation - stronger gene-activation in hippocampus and retrosplenial cortex following spaced than massed training. In a subsidiary study, a separate group of animals replicated aspects of this training profile, going on to show enhanced memory when training was subject to post-encoding environmental novelty. Distinctive features of this protocol include its potential validity as a model of memory encoding used routinely by human subjects everyday, and the possibility of multiple within-subject comparisons to speed up assays of novel compounds.

INTRODUCTION

Transient memories are formed as we go about our daily life at home or work. They generally occur against the background of relevant prior knowledge. Many of these ‘everyday’ memories, being of little consequence, are forgotten rapidly as Ebbinghaus first reported with experiments on memory training for nonsense syllables (Ebbinghaus 1885). However, a subset of everyday memories may be retained overnight or for longer (Wixted 2004). What is responsible for the selectivity of daily memory? Might better understanding of the relevant neural mechanisms be helpful for the development of effective cognitive enhancers? This methods report/technical spotlight article highlights an optimized protocol for monitoring everyday memory in animals. Its development was, in part, inspired by the “Rivermead Everyday Memory Task” used in clinical practice and research (Wilson, Cockburn et al. 1991).

Memory of successive items over time reflects variations in the effectiveness of encoding and/or retention of memory. Synergistic or competitive effects have long been studied, such as the classical phenomena of retro- or proactive interference (Muller and Pilzecker 1900, McGeoch 1932, Underwood 1969). Neurobiological studies have identified changes in memory retention caused by ongoing events at the time of encoding (such as changes in attention or metaplasticity: (Abraham and Bear 1996, Kentros, Hargreaves et al. 1998), by events shortly before or after encoding that trigger plasticity protein-synthesis (synaptic tagging and capture: (Frey and Morris 1997, Redondo and Morris 2011), and by neocortical mechanisms engaged for long time periods after encoding (such as systems consolidation: (Squire 1992, McGaugh 2000, Dudai and Morris 2001). Alterations in the effectiveness of memory retrieval can also occur in association with differential access to appropriate retrieval cues (Tulving 1983, Spear and Riccio 1994). To understand the neural dynamics of everyday memory processing in more detail, we need better, theoretically guided animal models. We now report a new and potentially useful model.

Two key principles have guided our approach. The first is that, just like humans facing daily memory challenges, the animals should not be ‘experimentally naïve’. A strikingly high proportion of behavioural neuroscience studies use naïve animals, many for good reason but many out of habit. Memory in experimentally naïve animals is frequently tested in tasks such novel object recognition, conditioned taste aversion, or various paradigms of fear conditioning. In this study, we deliberately allow memory

processing to occur against a background of experimentally controlled prior experience that permits repeated novel encoding and retrieval across days. This background likely affects the efficiency of encoding or consolidation (or both). A second principle was to develop a protocol in which what is remembered from one moment to the next is usually but not always forgotten. This is not only more ‘realistic’ of everyday life, where we forget a great deal, but also motivated by a specific theoretical model of memory formation based on the time-course of activity-dependent synaptic plasticity (Morris 2006). While various widely used ‘everyday’ tasks could be included in such a protocol, including recognition memory, our starting point was spatial memory – analogous to the “where did I leave my glasses?” kind of memory (as examined in the Rivermead Test). Training took place everyday, over weeks and months in a repetitive manner, with only occasional tests of the memorability of a specific item.

The ‘delayed matching-to-place’ protocol in the watermaze (Steele and Morris 1999, da Silva, Bast et al. 2014) is an example of an everyday memory task as defined, but an appetitively motivated alternative is more realistic than a task involving escape from water. We used an ‘event arena’ in which rats are trained to dig for food in sandwells whose location varied from day to day. Each day, for memory encoding, they explore the arena to find one or two sample sandwells (of which only one sandwell was rewarded), both located in a virtual array of up to 43 locations across sessions. For memory retrieval, at an interval of 60-90 min later, a rewarded choice trial was typically given with 6 sandwells of which only the correct one from the sample trial was rewarded. Win-stay training continued like this over many days and weeks as the animals first learned the task and then performed it well – learning anew each day and rapidly forgetting where the food had been over the preceding days. Apart from daily choice-trials, memory was also tested occasionally using an analogous technique to the watermaze - non-rewarded probe tests at various delays up to 24 h. In these, the time spent digging at each of 6 available sandwells, none of which contained accessible food during the test, was carefully measured.

Such a protocol involves new spatial memory encoding everyday – where the ‘event’ is learning where to dig each day and then rapidly forgetting where the food had been over the preceding days (Bast, da Silva et al. 2005). With modest reward on the sample trial, delay-dependent forgetting results in the memory of the daily location of food being at chance levels within 24 h. The present protocol has the important

refinement that the sample protocol has two sandwells rather than just one. Pilot work revealed that this reduced variability because the animals learn not to keep digging at one Sandwell, but to switch between sandwells as an optimum strategy to secure reward during both memory encoding and retrieval. Aspects of the encoding protocol were then systematically varied in ways that served to calibrate the new task (single vs. multiple rewards, the temporal spacing of multiple rewards) to identify whether these would affect memory retention. To explore the future potential of the new technique, RT-qPCR and RNA-Seq analyses were conducted to examine training induced gene-induction (Sheng and Greenberg 1990, Lanahan and Worley 1998). Assessment of the effect of a phosphodiesterase4 inhibitor, the putative cognitive enhancer Rolipram (Rutten, Prickaerts et al. 2006) further served to validate the behavioural protocol. A separate cohort of animals was trained in a subsidiary study to establish a replication and to explore the impact upon retention of post-encoding novelty that is known to drive immediate-early and plasticity-protein synthesis (Guzowski, McNaughton et al. 1999, Guzowski, McNaughton et al. 2001, Wang, Redondo et al. 2010, Takeuchi, Duzskiewicz et al. 2016).

METHODS

Subjects

The main study used 24 adult male Lister Hooded rats weighing 200g-250g on arrival. They were approximately 2 months old and training took place over a period of 4 months; all but one rat completed the testing (data is reported on n=23). They were group housed (3-4/cage; 12-h light-dark cycle; all training in the light phase). After habituation to the animal facility with free feeding and regular handling, they were food restricted to maintain body weight at 90% of the free feeding weight throughout the training and testing period. If an animal's weight dropped below 85%, it was individually fed to gain >85% body weight. The subsidiary study used 11 adult male Lister Hooded rats (250 g on arrival) maintained identically. All animal experiments were performed in accordance with the regulations and guidelines of the UK Home Office regulations (PPL number, 60/4566).

Apparatus – the event arena

All experiments were conducted using an 'event arena' (1.6 m x 1.6 m) whose floor surface consisted of 49 square Plexiglas panels (20 x 20 cm). Some of these panels had holes of 6 cm diameter into which sandwells could be placed (Fig. 1A, Fig. S1A). The floor panels were cleaned regularly and replaced in different locations to randomize any residual olfactory bias. There were prominent 2D and 3D extra-arena cues (Fig. S1B), and two intramaze landmarks (square-tower in row 4, column 2; golf-ball tower in row 4, column 6). The arena had transparent side-walls (30 cm high), and four start-boxes at the center of the North (N), S, E and W walls made of black Plexiglas (transparent lid, carpeted with sawdust, available water cup, and automated doors, and thus darker inside than the main arena). A ceiling mounted videocamera was used to monitor animal movements and choices, and this connected to a DVD drive and a LabView event-recording system.

Insert Figure 1 about here

Plexiglas sandwells (6 cm diameter, 4 cm depth), that contained hidden food pellets, were placed in the floor panels with holes (Fig. 1B, Fig. S1C). To mask the smell

of the reward, the sandwells were filled with bird sand mixed with Garam Masala (P&B (Foods) Ltd., 150 g/ 5kg sand initially, with 3g / 5 kg added every session (to replenish the masking effect, and the sand lost during cleaning). Each sandwell had a spherical plastic bowl at the bottom in which reward pellets (5 x 0.5 g) were placed to be accessible ('rewarded sandwell'). This plastic bowl also made it possible for an equal number of reward pellets to be placed underneath, and thereby inaccessible ('non-rewarded sandwell'). The plastic bowls had holes and so were porous to odors, ensuring that the rewarded and non-rewarded sandwells, not only contained the same number of reward pellets at approximately the same depth in the sand but would also smell the same (Fig. 1B, Fig. S1C). The sandwell was designed with an extra panel at the bottom to enable easy changing of the bottom pellets, with fresh pellets used in every session. Extensive randomizing and counterbalancing was conducted to minimize any olfactory artifacts; this included ensuring that the sandwells used in the sample trial were not used for the choice trial of the same session; wiping the arena floor with an alcohol impregnated towel between each animal trial, and before choice and probe trials.

Behaviour training, sample trials and choice trials

There was a series of distinct phases of training: (1) habituation, (2) training to dig, (3) main training sessions with sample and choice trials, and interspersed tests involving (4) within-subject manipulation of diverse parameters that might affect the strength or persistence of memory (e.g. spacing of reward availability in a sample trial) with associated memory probe tests.

Habituation (session -8): The animals, at 90% of free-feeding body weight, were brought to the arena and placed in the start-box for 30s, and then allowed to explore in the arena for 10 min, before being placed back in the start-box for 30s, and then given a reward pellet in the home cage (Fig. 1C). *Training to dig (sessions -7 to -1):* The animals were given one pellet in the start-box, then allowed to explore to look for the reward pellets (BioServ, Supreme Mini-Treat 0.5 gm) at the single sandwell placed in a random location of the arena, and taught to bring the pellet, one at a time, back to the start-box of the day to consume 3-5 pellets (Fig. 1C). Pellets were initially visible on the sand, and then buried lower day by day, encouraging the animals to dig deep to find the pellets at the bottom of the bowl. By the end of habituation sessions, all the rats had learned to dig

with conjoint experience of the sandwells in the home cage. They also started to learn the win-stay strategy of returning to a rewarded sandwell that was explicitly trained during the subsequent sessions.

Training sessions with sample and choice trials (sessions 1-81): Training then commenced, and repeated across days, 5-7 days per week, for several months. A training session consisted of a sample trial (ST = *memory encoding trial*) and, 60-90 min later, a choice trial (CT = *memory retrieval choice trial*) (Fig. 1D). On ST, the rats were presented in the arena with one rewarded sandwell (sessions 1-18) or two sandwells of which only one contained accessible reward (session 18 onward). The rats were first given a food pellet (0.5g) of a specific flavor in the start-box. The door was then opened permitting access to the arena where the rat could find and dig up the reward pellet that it then immediately, or with gentle encouragement for the first few trials, took back to the start-box to eat (Whishaw, Kolb et al. 1983). Thereafter, between 1 and 5 pellets were available during an ST, as described in later protocols. On CTs, the rats were presented with 6 sandwells in the arena, with the correct sandwell being in the same location as the rewarded sandwell of the earlier ST (thus, the correct sandwell is called the ‘cued’ sandwell in CT and PT although this is a memory cue; there is no explicit sensory cue marking it) (Movie S1).

Each floor panel with a 6-cm hole for sandwell placement could be placed in any slot in the arena, the only constraints being that the locations of the intramaze cues (Row 4, Columns 2 and 5) and those directly in front of each of the 4 possible start-box locations were never used (i.e. 43 possible out of 49). The sandwell locations (map) changed across sessions to exclude procedural searching strategies such as right/left, close/far, specific quadrants, centre/corner, or distance from the intramaze cues. The rewarded and non-rewarded locations were counterbalanced across animals within a day. Twelve different flavors of reward pellet were also used, to help discriminability and so potentially to enhance memory (these flavors were curry, blueberry, bacon, nutmeg, anise, maple, garlic, ginger, cinnamon, mustard, coffee, and peach). Flavors were repeated only after 12 sessions.

Behaviour was carefully monitored throughout for errors and correct-digs from which a performance index (PI) and Latency were calculated. On CTs, the experimenter noted the identity and number of sandwells at which an animal dug (errors) before digging at the cued sandwell (correct dig), and the time before digging at the cued

sandwell. The chance level for errors is 2.5, but we aimed in this 6-alternative forced-trial procedure for a stable level of performance at around 1 error/trial. The PI was calculated as $100 \times (5 - \text{errors} / 5) \%$, for which chance is 50%. The latency to 'first-dig' at the correct sandwell in the CT dropped gradually to a stable level of <20s (Fig. S1D), excepting on the single control session (S60). (See results section for details.) This was a session to check for olfactory artifacts by deleting the sample trial and running only the choice-trial. It was expected that performance would be slow and at chance.

Probe trial sessions

Once stable performance was reached, occasional Probe Trial (PT) sessions were interleaved with further S+CT training – these being associated with variations in protocol for the ST on a within-subject basis (see below). These sessions consisted of an ST and then, after a memory interval, the PT itself. All 6 sandwells had inaccessible food pellets (Nonrewarded sandwells in Fig. 1D). The animals could dig at one or more of the sandwells, but reward was inaccessible at every location. Effective memory was preferential digging at the previously rewarded sample location (cued location). The rats were given one pellet (0.5g) in the start-box, and then allowed 60 sec in the arena (Movie S2). Digging time was defined as direct contact with the sand with the forepaws; in contrast, sniffing, a nosepoke, or a gentle foot-print associated with only a visit were not considered as 'digging.' The digging time scoring (0.1 s resolution) was performed in blind manner by the single experimenter (MN, main study; MC, subsidiary study), and checked with further off-line blind scoring by an independent observer (RF, see Fig. 1F for one example of experimenter/observer cross-correlation). After 60 sec, the door of the start-box was closed and the rats returned to it by hand. To reduce 'extinction', the experimenter then placed 3 accessible pellets at the bottom of the sandwell at the correct location in the arena, opened the start-box door, and the rat was again allowed to find and retrieve all 3 pellets. All probe tests were interleaved with two or more regular training sessions (STs followed by CTs) (Fig. 1D).

Memory parameters examined behaviourally and biochemically

Once the animals had reached stable asymptotic performance on daily ST and CT sessions, a series of PTs were conducted preceded by distinct manipulations on or after the daily sample trials (STs).

*First, **time delays**:* we examined memory duration – scheduling the PT in a counterbalanced manner 24 min, 2.4 h, and 24 h after an ST in which the animals were rewarded with a single 0.5 g food pellet. This and future tests also served to check on the accuracy of memory scoring during the PTs.

*Second, **massed vs. spaced**:* we compared the classic parameter of massed vs. spaced access to reward on ST, with 3 pellets available on each ST. After retrieving the first reward pellet, and returning to the start-box to eat it, the start-box door was closed. The door was then re-opened for access to the two further pellets after either 30 s (massed) or 10 min (spaced access) – these access delays being scheduled within-subjects in a counterbalanced manner. After collecting and eating the third pellet in the start-box, the animals were returned to the home cage. Between rewards, the experimenter quickly cleaned any sand dug up by the animal. This cleaning process was introduced to limit the emergence of any strategy involving preferential digging at a sandwell surrounded by sand, rather than guidance by spatial memory (Movie S3).

*Third, **biochemical assay for task-associated gene expression**:* we looked at the impact of memory encoding on gene expression. A sample trial was given using either the massed or spaced training protocol with total of 3 reward pellets retrieved. We considered carefully the appropriate protocols and time-delays before sacrifice for conducting this assay, deciding to keep the interval after the 2nd reward pellet consistent across conditions (see Fig. 4A). This means that, as a confounding of the interval between reward pellets, there is longer in the start-box for the massed condition, and this may have, in some way contributed to the differential pattern to be reported. There are several potential ways to address this problem, but all involve added difficulties (e.g. equating time in the start-box by taking the animals in and out; leaving the animals in the start-box for a long period after the sample trial before returning to the home-cage, which would have added novelty. We chose the simplest protocol. Thus, forty-five (45) min after the 3rd reward pellet, the animals were briefly anaesthetized in an isofluorane chamber and decapitated. The brains were rapidly taken out and placed in ice-cold PBS for 30s. A coronal block (A/P from -2 mm to -7 mm from Bregma) was cut using a chilled brain matrix, dorsal hippocampus and retrosplenial cortex were dissected, and snap frozen in the liquid nitrogen. The samples were shipped to Dart NeuroScience, and then analyzed using RT-qPCR and RNA-Seq (see below).

Fourth, cognitive enhancement: prior to (3) above, we looked at the impact of i.p. injection of a phosphodiesterase 4 inhibitor (PDE4 inhibitor) Rolipram at two time points after memory encoding. The drug solution was freshly prepared on the day of injection in the same way as described by Rutten et al. (Rutten, Prickaerts et al. 2006). Rolipram (Tocris, #0905) was first dissolved in ethanol, with injectable grade water added to during rigorous vortexing. This solution was then mixed with 2% Tylose (Sigma, MH 300, #93800) in water that had been prepared by overnight gentle mixing on a rotator at 4°C. A final concentration of 68.1 mM (0.019 mg/mL) in 5% Ethanol/1% Tylose was created, with 0.03 mg/kg injected intraperitoneally in the control room of the laboratory at 45 min or 3 h after the ST (2-sandwell procedure, one pellet retrieval).

Fifth, novelty exposure: as brain tissue was taken in step 3 above to measure differential gene expression, the impact of novelty on retention was tested in the subsidiary study. Environmental novelty involved placing the animals for 5 min into a large square Perspex box 30 min after a daily ST (post-encoding novelty). The box measured 1.4 m square, and contained different floor surfaces on successive visits. Rats are very sensitive to the surface on which they walk and the use of very different surfaces to that of the main event arena evoked activity and exploration on each occasion, provided these are not scheduled too frequently. Two conditions were compared – one pair of tests in which either novelty exposure or remaining the home-cage followed 30 min after an ST with 1 pellet reward (Low reward); and another with 3 pellets reward (High reward). The two-sandwell protocol was used throughout for which 1 pellet reward is insufficient to produce much more than a transient memory.

RT-qPCR and RNA-Seq

RNA Extraction: Frozen tissues were homogenized in Qiazol (Qiagen) using Lysing Matrix D ceramic spheres in tandem with FastPrep 24™ 5G homogenizer (MP Biomedicals). RNA extraction wash steps and elution were performed robotically with Qiacube (Qiagen) with an RNeasy Mini kit (Qiagen) and DNase treatment (Qiagen) as per manufacturer's instructions.

Quantitative PCR (qPCR): The DNase treated RNAs were reverse transcribed with random hexamers (Taqman Reverse Transcription Kit, ThermoFisher Scientific). qPCR reactions were run in triplicate using the Taqman Fast Advanced Master Mix (ThermoFisher Scientific) with Taqman probes for the immediate early genes, *Arc*, *c-Fos*,

Npas4 and the reference housekeeping genes *Tbp*, *Gapdh*, and *Actb* (ThermoFisher Scientific) on a StepOnePlus Real Time PCR System (ThermoFisher Scientific). We performed 40 cycles of PCR: 1X (50°C for 2 min, 95°C for 20 s) and 40X (95°C for 1 s, 60°C for 20 s), and determined relative mRNA levels by normalizing cycle threshold (C_t) values of the target and reference housekeeping genes using the $\Delta\Delta C_t$ method. Briefly, we calculated ΔC_t (the difference in C_t between target genes and reference genes) for experimental and control conditions. The difference between these ΔC_t values for control and experimental conditions is the $\Delta\Delta C_t$ and changes in mRNA levels are directly proportional to $2^{-\Delta\Delta C_t}$.

RNA-Seq: RNA concentration and quality were measured with a Qubit Fluorometer (Life Technologies) and a Bioanalyzer (Agilent), respectively. RNA-Seq libraries were prepared with non-stranded NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB). Briefly, mRNA was purified from 200 ng total RNA and then fragmented at 94°C. cDNA was prepared with random hexamers. Illumina-compatible adaptors and unique indexes were added and DNA fragments were amplified with 12 rounds of PCR. Ampure beads (Beckman Coulter) were used for size exclusion. Libraries were run on the Bioanalyzer and had mean fragment sizes between 384 and 457 bp. 3-20pM of each library underwent paired end (2x50) sequencing on a HighSeq 2000 (Illumina) with 8 libraries multiplexed per lane. FastQ files were aligned to the Ensembl transcriptome via Bowtie and differential expression was determined using DE-Seq. To avoid complications from false positives, we set a low p-value cut-off of $p < 0.0001$ for significance and focused our analysis on those hits that were concordant between hippocampus and retrosplenial cortex.

Randomising, Counterbalancing and Statistical Analysis

Behavioural experiments require careful attention to detail with respect to both their conduct and analysis. Points to note include that the comparisons of conditions are all within-subject such that there is no issue of randomising the allocation of animals across groups – there is only one group in both the main and subsidiary studies. Counterbalancing is also simple in principle but complicated in practice, the aim being to ensure that, for any one of the four start-box positions that are used across days, half the animals are trained on each day with the cued sandwell in a near location and the other animals in a far location. Similarly, the placement of the non-rewarded sample

may be near or far, and to the left or right of the rewarded (correct) sample location. With respect to statistical analysis, the data always complied with assumptions of homogeneity of variance allowing parametric ANOVAs to be used throughout. Some analyses focused only on choice performance using the Performance Index (PI), while others focused on (1) time spent digging at the rewarded location (hereafter 'cued location'), or (2) the relative digging time at the cued location, the non-rewarded sample location (non-cued trained) and the remaining incorrect sandwells (non-cued untrained locations). Note the term 'cued' does not refer to any stimulus marking the rewarded location; to the contrary, all cueing is within memory. As we used a within-subjects design, Greenhouse-Geisser corrections of degrees of freedom were routinely used within SPSS to ensure a conservative approach to the analysis, but their use leads to somewhat 'odd' values for degrees of freedom, as apparent below.

RESULTS

The rats rapidly learned to run from the start-boxes into the arena and to dig for food that they would always carry back to the (darker) start-box to eat. Following habituation during which, as noted in the Methods, some learning would have occurred, we began training with a single sample sandwell as the encoding trial (ST) followed by a choice trial (CT), but switched after 18 sessions to using two sample sandwells of which only one was rewarded (Fig. 1E).

Performance on the 6-sandwell CTs, circa 1 h after the sample trial, was measured using the performance index (PI; see Methods). This was already just above chance in some animals after habituation, but then assumed by all animals and sustained at an average level of 77.9% from session 10 onwards with a mean z-score relative to chance of 4.8 (Fig. 1E). This remarkable stability of the PI across 3 months of testing is important as it constitutes the background against which the successive daily 1-trial encoding can be evaluated. On session 60, the control condition was scheduled in which the ST was not given but a CT still run; the animals should then *not* know which sandwell was correct and should fall to chance. The 'virtual' correct sandwell location was determined according to the same mapping rule as in regular training – namely that any new rewarded sample sandwell location could not be in the same quadrant in the arena as that of the previous session. All the condition biases - left/right from the startbox, corner/center, near far from the startbox - were counterbalanced among 4 sub-batches within the session. In such design, the performance index of session 60 was calculated using the 'virtual' error. Virtual error is the number of 'virtual' errors made before digging at the 'virtual' correct one (which was in practice rewarded). An analysis of the PIs from session 58 to 62 shows that, as predicted, performance dropped to chance on session 60 (repeated measures (RM)-ANOVA: $F = 9.52$, $df\ 2.9/61.2$, $p=0.000036$; degrees of freedom adjusted by use of Greenhouse-Geisser correction; one-sample t-test vs. chance: $t_{22} = 1.16$, $p=0.26$ for session 60 and $t_{22} > 4$, $ps < 0.0001$ for all other sessions; Fig. 1E). Interspersed PTs (gray shading) were thereafter used as the primary measure of memory. The scoring during PTs was double-checked for a sub-set of animals using independent blind-scoring. A very high cross-correlation ($R^2 = 0.992$) was observed between the initial semi-blind score derived from scoring during testing (by MN) and that derived from off-line blind analysis of DVD recorded data by an independent observer (RF; Fig. 1F). Thus, the rats successfully encoded the location of

the rewarded sandwell during an ST (with one or two sample sandwells); this encoding was necessary to make a correct choice during the succeeding CT; and PT scoring was objective.

The use of a single sandwell (containing 1-5 reward pellets) as the sample trial (ST) had the unintended effect of encouraging, during CTs and PTs, persistent digging at whichever sandwell was chosen first. As this included trials when errors were made, persistent digging at an initially selected location resulted in high variability of test scores. The switch to using 2 sandwells on STs resulted in a strategy change to frequent shifting between sandwells to secure reward. This greatly reduced error variability during later PTs at the cost of a slight decrease in average time spent digging at the correct (cued) sandwell.

Delay-dependent forgetting was observed after sample encoding using both the 1- and 2-sandwell procedures, each involving a single small reward (1 pellet). Two counterbalanced series of 3 within-subject PTs (no food available) were conducted at delays of 24 min, 2.4 h and 24 h after STs, with 2 intervening regular training sessions (consisting of S and CTs, Fig. 2A). Memory was measured in terms of the percent time spent digging at the correct (cued) vs. the incorrect (non-cued) sandwells during the PTs (Fig. 2B-C). The overall pooled repeated measures ANOVA of the two sets of data showed significant delay-dependent forgetting over 24 h ($F = 3.87$, $df\ 1.6/35.7$, $p=0.038$); Greenhouse-Geisser correction for repeated measures), with a strong downward linear trend over time ($F = 10.12$, $df\ 1/22$, $p=0.0043$). Subsequent t-tests comparing correct (cued) sandwell performance at each memory interval indicated above chance performance in the PTs after both 1-sandwell and 2-sandwell sample trials at 24 min and 2.4 h (1 sandwell: $t_{22} = 5.12$ and 4.95 , $ps = 0.00004$ and 0.00006 , respectively) for both; 2 sandwell: $t_{22} = 4.34$ and 3.24 , $ps = 0.00027$ and 0.0039 but chance performance at 24 h (1SW $t_{22} = 1.96$, $p = 0.063$; 2 SWs $t_{22} = 1.73$, $p = N.S.$).

Insert Figure 2 about here

The impact of massed vs. spaced access to reward was then examined, using only the 2-sandwell procedure for STs (Fig. 3A). Memory retention improved when reward availability was increased from 1 to 3 pellets, with either massed access to reward (inter-reward-interval of 30 sec) or spaced access (IRI=10 min) (Movie S3).

However, while massed reward produced good memory at 2.4 h, there was still forgetting over 24 hr. In contrast, spaced reward had a dramatic effect on 24 h memory which was both above chance at 24 h and not measurably different from that at 2.4 h (Fig. 3B). Importantly, the ANOVA comparing massed vs. spaced reward and time-intervals revealed a significant interaction ($F = 7.39$, $df\ 1/22$, $p = 0.013$). Separate t -tests showed that memory was above chance at 24 h only for 3 spaced rewards (Spaced: $t_{22}=4.52$, $p = 0.00017$; Massed: $t_{22}=1.73$, $p = 0.099$), whereas location memory at 2.4 h did not differ after massed vs. spaced reward (Massed = $35.3 \pm 3.3\%$; Spaced = $32.1 \pm 2.7\%$; N.S. $p = 0.45$). Thus, the impact of spaced reward seems to be on the *persistence* of everyday memory at 24 h rather than immediate memory strength at 2.4 h.

Over the 3 rewards of the ST, we also examined the rate of decline of errors in choosing between the 2 sandwells (Fig. 3C). To be expected, the animals were at chance for the 1st reward pellet, but improved for pellets 2 and 3. If anything, the trend was for this improvement to be slightly greater for massed than spaced trials, but this did not reach significance ($F=1.15$, $df\ 1/22$, $p=0.295$). However, the improvement across successive pellets (1st, 2nd and 3rd) was significant ($F=48.51$, $df\ 2/44$, $p = 7.4E-12$).

Insert Figure 3 about here

Because memory consolidation requires protein synthesis and transcription (Davis and Squire 1984, Alberini and Kandel 2015), we then examined whether daily encoding of spatial memory resulted in changes in gene expression, and more interestingly, if there were differences in the response to massed and spaced training. At the end of study, rats were given either massed or spaced access to the reward presentations in the 2-sandwell protocol and euthanized 45 min after the second of three trials (Fig. 4A; an interval chosen to minimize any possible impact of slight differences in timing between paradigms). The three groups (Control (no ST given and Naïve), Massed, and Spaced) were assigned so as not to differ with respect to prior task performance (PIs for Ctrl, Massed and Spaced were 77.4, 77.8, 77.5 %, respectively (one-way ANOVA, N.S.); and PT average scores were 30.1, 29.4, 28.6 %, respectively (also N.S.)). Number of subjects = 11, 8, 8 (Note: The naïve rats ($n = 4$) did not differ from the trained animals ($n = 7$) given no-ST prior to euthanasia and the RT-qPCR analyses, so pooled together as a control group (Ctrl $n=11$)). Our analysis focused on the dorsal

hippocampus and the retrosplenial cortex - two regions that are critical for spatial navigation and memory (Vann and Aggleton 2002, Frankland and Bontempi 2005, Vann, Aggleton et al. 2009)(Fig. 4A).

Initially we performed RT-qPCR on three immediate-early genes (IEGs), *Arc*, *c-Fos* and *Npas4* as well as three housekeeping genes (HKP), *Gapdh*, *Actb* and *Tbp*. All three IEGs were markedly upregulated by massed and spaced training in both tissues, whereas the levels of the housekeeping genes were unchanged (Fig. 4B-C). The $\Delta\Delta Ct$ was calculated with normalization to *Gapdh*, but similar data was observed when either of the other two housekeeping genes (*Actb* and *Tbp*) was used as a reference. The data from all three IEGs was pooled, and that for the three HKP genes, and an RM-ANOVA conducted across the three conditions (Ctrl, Massed, Spaced). This revealed a highly significant IEG vs. HKP x Conditions interaction in both brain regions (HPC: $F=24.08$, $df\ 2/24$, $p = 0.000002$; RSC: $F=14.13$, $df\ 2/24$, $p = 0.000088$). This justified looking at the IEGs alone, revealing significant effects of training condition, i.e. between Control, Massed and Spaced conditions (HPC: $F=19.64$, $df\ 2/24$, $p = 0.000009$; RSC: $F=13.17$, $df\ 2/24$, $p = 0.000138$). Subsequent orthogonal comparisons showed that, for HPC, the Control condition was significantly lower than the combined massed and spaced conditions ($F = 34.1$, $df\ 1/24$, $p < 0.001$) and that activation with spaced reward was higher than with massed ($F = 5.12$, $df\ 1/24$, $p < 0.05$). Based on these data, we decided to perform RNA-Seq experiments on the same samples.

To ensure that our RNA-Seq data provided accurate measurement of mRNA, we plotted the normalized FPKM values calculated with RSEM for the genes already tested with qPCR (Fig. 4D-E). We found that the RNA-Seq and RT-qPCR methodologies gave nearly identical results, and proceeded to measure the effects of training on all protein coding genes using DE-Seq2. In the massed condition, the expression of 45 genes was increased in HPC and 82 in RSC. Of these genes, 29 were significantly induced in both structures. In the spaced condition, we found 113 upregulated genes in HPC and 137 in RSC - with 54 genes induced in both. Because any random false-positives in a given tissue would be exceedingly unlikely to replicate in independent RNA samples from another tissue, we examined further only the 29 'Massed' and 54 'Spaced' genes that were up-regulated in *both* the HPC and RSC, and found that of the genes meeting our strict criteria in massed training, 27 were also up-regulated in spaced (Fig. 4F). Because more genes were upregulated in the spaced condition than in the massed, we wanted to

determine if there were any genes only regulated by spaced food reward. We focused on the 56 genes that were significant in both tissues with either training paradigm and correlated the fold-change induced by massed and spaced training in HPC (Fig. 4G) and RSC (Fig. 4H). This analysis showed that the genes that were up-regulated significantly in rats trained with spaced trials were also up-regulated by massed training, but did not always reach our strict statistical significance cut-off. This indicates that the constellation of genes induced by massed and spaced training is similar. However, a linear fit of the data ($R^2 = 0.94$ and 0.96 for HPC and RSC, respectively) revealed that the relationship between the effects of massed and spaced trials followed a power law of 1.33 in both tissues, indicating a larger increase in gene expression following spaced food reward (Table S1). Only 2 of the 56 genes were increased to a greater extent in the massed condition and, interestingly, these two genes were significant in both HPC and RSC. In contrast, a higher number of genes were increased to a greater extent after spaced training. Orthogonal comparisons across all 56 genes revealed that 7 genes were significantly more induced in spaced than massed in both HPC and RSC, all of which are CREB-target genes; 14 genes were significantly more induced in spaced than massed in either HPC or in RSC; some of these are also CREB-targets. Amongst those, we identified several members of the *Egr* family of genes, *Dusp1* (a regulator of the MAPK pathway), and the heat shock proteins *Hspa1a* (Hsp70) and *Hspb1* (Hsp27), chaperones critical for the folding of newly synthesized proteins and cellular differentiation (Fig. 4G-H). These data indicate that the massed and spaced training paradigms initially recruit very similar gene expression responses, but that the *magnitude* of the response is greater with spaced training.

Insert Figure 4 about here – RT-qPCR and RNA-Seq

The impact of the putative cognitive enhancer Rolipram (0.03 mg/kg) was then investigated using two post-encoding injection times, 45 min and 3 h, using only single reward encoding in the ST to maximize forgetting over 24 h (Fig. 5A; but still using 2 sandwells with only one rewarded in the encoding trial). No enhancement was observed with the 3 h post-encoding injections, but a striking improvement was apparent at 45 min relative to vehicle injections ($28.5 \pm 2.8\%$, compared to $19.8 \pm 2.4\%$). An overall ANOVA of digging in the probe trials at just the cued-location of the sample

trial showed a trend in the interaction between Drug and Time-Delay ($F=3.87$, $df\ 1/22$, $p = 0.061842$). However, an RM-ANOVA comparing digging at the Cued-trained, Non-cued trained, and the Non-cued untrained sandwells was significant for the 45 min post-encoding injection ($F = 10.92$, $df\ 1.415/31.12$, $p = 0.001$), together with an interaction reflecting the impact of Rolipram compared to vehicle ($F = 3.67$, $df\ 1.615/35.54$, $p = 0.044$) (Fig. 5B). The analysis of performance with the 3 h post-encoding injections showed no drug effect ($F<1$), although the usual difference across sandwells ($F=9.36$, $df\ 1.42/31.24$, $p = 0.0019$). Thus, the retention of 'everyday' spatial memory can be enhanced by inhibition of PDE4 and such augmentation of memory is possible in behaviourally experienced animals.

Insert Figure 5 about here

The subsidiary study involved training a separate cohort of animals in same way to investigate the impact of 5 min of post-encoding environmental novelty on memory retention after weak (1 pellet) or strong reward (3-pellet, massed) in the 2-sandwell sample trial procedure (Fig. 6A). The excellent stability of performance on Training Days is shown in the Fig. S2. After an initial learning period, daily performance was at or around 75-85% throughout a long series of memory probe tests (Chance performance is 50%). This stability gives confidence that probe tests conducted early or late in training were against a stable performance baseline.

As shown in Fig. 6B-C, for the strong protocol, there was excellent memory for the rewarded sample location at 24 min ($58.2 \pm 7.1\%$; chance = 16.7%). Memory declined over 24 h to chance ($25.6 \pm 6.3\%$) as expected for massed reward (an 'internal replication'). However, if 5 min novelty exposure was scheduled 30 min post-encoding, memory was sustained above chance ($39.2 \pm 4.0\%$). The data plotted is an average of the first 60 sec searching, during 2 separate probe tests for the 24 h tests, with only one test being sufficient for the 24 min test. The RM-ANOVA of digging across the sandwell locations showed a within-subjects conditions effect ($F = 6.72$, $df\ 1.97/19.70$, $p = 0.0061$) that declined in a monotonic fashion across conditions (24 min, 24 h+Novelty, 24 h-only: Linear $F = 11.89$, $df\ 1/10$, $p = 0.062$). Thus, retention was greater with post-encoding novelty. Separate tests established that performance was above chance in the 24 min and 24 h+Novelty conditions ($p = 0.00017$ and 0.00022 , respectively), but at

chance for the 24 h condition ($p = 0.19$, NS). Novelty scheduled 30 min after encoding appeared to improve the ability of the animals to discriminate the rewarded and non-rewarded sample locations (Fig. 6C; plotting % time spent at the Non-cued trained location from that at the Cued location, while ignoring the other Non-cued untrained locations. Specifically, this discrimination is clear 24 min after encoding but at chance after 24 h. However, post-encoding novelty lifts the capacity to distinguish Non-cued trained from Cued-trained at the 24 hr test interval (ANOVA revealed a decline across memory delays ($F = 4.16$, $df\ 1.99/19.95$, $p = 0.031$), declining monotonically (Linear $F = 7.98$, $df\ 1/10$, $p = 0.018$). Subsequent planned orthogonal comparisons revealed that the 24 min and 24 h+Novelty conditions did not differ for the Cued location ($F = 2.01$, $df\ 1/20$, N.S.), but the mean discrimination between the two sample locations in these two conditions was significantly greater than that in the 24 h only condition ($F = 6.29$, $df\ 1/20$, $p < 0.01$).

Separate probe tests were conducted using the weak 1-pellet reward protocol (Fig. 6D, E). These revealed modest memory at 24 min, nothing at 24 h, and no impact of novelty on either absolute levels for each sandwell or with respect to the difference score (ANOVAs: $F = 1.66$, $df\ 2/20$, $p = 0.216$ (N.S.); and $F = 2.69$, $df\ 2/20$, $p = 0.092$). Taken together, these findings imply that post-encoding novelty can augment retention but there has to be some minimal level of memory for this to be effective.

Insert Figure 6 about here

DISCUSSION

This report/technical spotlight reports an everyday memory protocol that enables 1-trial memory to be investigated repeatedly over months in experienced animals thereby enabling within-subject comparisons. In this study, we have conducted both ‘calibration’ experiments in relation to well-established principles of memory, and a ‘prospective’ study illustrating its potential for future work. By way of calibration, we observe: (1) delay-dependent forgetting of one-trial appetitively motivated spatial memory over 24 h; (2) more persistent memory over time (i.e. better retention) with multiple spaced access to reward but not massed access to reward; (3) enhanced retention when the PDE4 inhibitor Rolipram was administered shortly (45 min) after

encoding. With respect to its ‘prospective’ use for future discovery research, we also report (4) upregulation of specific IEGs and a wider pattern of gene-induction in association with everyday memory training that is quantitatively greater for spaced compared to massed experience. To our knowledge, this is the first report of such differential gene expression as a function of trial spacing in mammals. These data were secured against a background of demonstrably stable levels of choice performance (our Performance Index (PI) measure), with the objective scoring of the Memory Probe Tests (PTs) secured by means of two independent but well-correlated observers. The reliability of the task was replicated in the second cohort of animals who went on to display, using the 2-sandwell protocol, that (5) post-encoding novelty enhances retention. We consider these results in relation to the value of our ‘everyday memory’ protocol, its likely generality, and thus its potential for making discoveries with respect to cognitive enhancement.

Everyday memory is characterized by substantial daily forgetting

David Marr suggested, as an assumption within his mathematical model of ‘archicortex’ (Marr 1971), that humans may encode a maximum of 1 memory per sec throughout the day, with overnight consolidation into neocortex, thereby requiring a maximal daily storage capacity in hippocampus of 10^5 - see (Willshaw, Dayan et al. 2015). This assumption, while likely inaccurate in detail, serves as a reminder that the mammalian episodic-like memory system should be capable of processing numerous events through the day with minimal interference. This idea is captured in several behavioural tasks, including spatial memory as tested here ((Olton, Becker et al. 1979, Steele and Morris 1999, Bast, da Silva et al. 2005), and recognition memory tasks as developed for non-human primates (Mishkin and Delacour 1975) and its rodent equivalent (Ennaceur and Delacour 1988). Our approach to ‘everyday memory’ incorporates the idea that distinct events can be encoded, remembered and forgotten each day including such information as “what, where, when, what context” (Griffiths, Dickinson et al. 1999, Eacott and Easton 2007).

Against the background of stability across testing, our first finding – delay-dependent forgetting of 1-trial memory over 24 h - captures a defining feature of this model of everyday memory. Our use of the 2-sample sandwell procedure extends the earlier observation of delay-dependent forgetting with a single sandwell (Bast, da Silva

et al. 2005), but now using a within-subjects protocol run everyday for 4+ months. We can think of the protocol as one that includes daily events that might be automatically remembered – being placed in the apparatus, waiting for the start-box door to open, leaving the start-box, searching, digging, finding food, returning to the start-box, and eating the food. We cannot test for each of these ‘everyday’ memories, but spatial memory in the arena serves as an index. During training sessions, the animals are given the opportunity to choose between 6 sandwells approximately 1 hr after encoding. Behaviourally, they display a ‘win-stay’ choice correctly from very early in training (there were 7 days of ‘habituation’ preceding that helped teach this strategy), eventually reaching performance of around 1 error per trial throughout the remainder of training (circa 80%). The stability of performance is reflected in a remarkable z-score, relative to chance, of >4.5 , that was shown, using a suitable control condition, was not due to any ‘olfactory’ artefact. When the choice-trial was occasionally substituted by a probe test in which reinforcement was not available for 60 sec, they show strong preferential digging at the correct sandwell at 24 min and at 2.4 h, but memory declined to chance over 24 h. The 2-sandwell sample procedure results in notably low variability in probe trial performance across animals as they acquired the sandwell-shifting strategy in the daily STs as discussed above. The stability of performance (Fig. 1E) and the sustained motivation of the animals should enable reliable within-subject comparison of drugs regardless of the sequence of drug testing over time. Performance stability was replicated in the subsidiary study (Fig. S2).

As further calibration of the task, we observed that memory improved with access to 3 spaced rewards on an encoding trial (i.e. the animal collects the 3 reward pellets one by one). The improvement of 24 h memory occurred with an inter-reinforcement interval (IRI) of 10 min but not when only 30 sec. This finding confirms a large body of findings indicating that spaced training produces better memory than massed training in humans and animals (Ebbinghaus 1885, Greene 1989, Commins, Cunningham et al. 2003), an effect also observed in studies of long-term potentiation (Kramar, Babayan et al. 2012) that likely reflects the need to engage long-term memory as well as working memory during acquisition. A potential complication is that the quantitative parameter of inter-reward interval (30 sec vs. 10 min) is confounded by total time spent in the start-box that may have effects on short-term satiation, arousal or frustration. However, with such a small proportion of the animal’s daily diet

consumed during the task, it was no surprise that the animals ate the pellets readily and within 30 sec and short-term satiation seems unlikely. In so far as arousal may have been affected, arguably the shorter interval would have sustained arousal better than the 10 min wait in the start-box, unless the longer interval of 10 min induced frustration. However, we suspect that the main contributor is that returning to the correct sandwell for the second and third reward pellet requires retrieval from long-term memory in only the spaced condition. Retrieval during an encoding session is known to enhance later memory – the so-called ‘testing effect’ (Karpicke and Roediger 2008) – and also causes differential memory-related gene expression.

Enhanced gene transcription associated with trial-spacing and enhanced memory

The prospective value of this new behavioural technique is shown in the observation of graded upregulation of IEGs genes such as *Arc*, *c-Fos* and *NPAS-4*, and heat shock proteins, *Per1*, and *Dusp1*, with no change in control ‘housekeeping’ genes in the hippocampus and retrosplenial cortex. Not only was gene-expression higher in animals trained in the task shortly before euthanasia, but was also significantly higher with spaced than massed training - a finding observed with both RT-qPCR and RNA-Seq. To our knowledge, this is the first study in rodents to find more gene expression in spaced over massed training. A potential qualification is that we tested gene-expression 45 min after the middle time-point of the sample trial, which constitutes a 9.5 min difference between the first and the last trials of massed or spaced training respectively. While this minor timing difference could possibly account for the differential expression of highly transient IEGs, it is unlikely that the many genes found in our RNA-Seq results should all show such time sensitivity. Interestingly, in the fruitfly *drosophila melanogaster*, memory encoded with a single session of olfactory conditioning is augmented by over-expression of the transcription factor CREB (cAMP-response element binding protein) (Yin, Del Vecchio et al. 1995). This finding was corroborated more recently by studies demonstrating that spaced, but not massed, training leads to CREB dependent activation of the calcium/calmodulin-dependent protein kinase II (CaMKII) and period genes in two dorsal-anterior-lateral (DAL) neurons in *drosophila*, and that protein synthesis in DAL neurons is required for LTM consolidation (Chen, Wu et al. 2012). In orthogonal experiments in mice, either spaced training or massed training combined with over-expression of CREB yields stronger fear memory than

massed training alone (Josselyn, Shi et al. 2001). Together, these results indicate that spaced training is associated with transcriptional activation in invertebrates and vertebrates alike.

Massed and spaced training trials both increased gene-expression, but the response was more pronounced after spaced encoding. The differential effects of massed and spaced training on signal-transduction cascades for gene-induction are poorly understood. However, individual trials may result in calcium signals that are already above threshold for triggering CaMKII, PKA or other signaling cascades. In such scenario, the spacing of reward would enable such cascades to be activated three times whereas, with massed trials, this may not occur due to a refractory period. As spaced training can sometimes compensate for the reduction of CREB (e.g. in CREB α/δ knockout mice (Kogan, Frankland et al. 1997, Bourtchouladze, Lidge et al. 2003)), there is the possibility that other pathways may be involved in memory persistence. Non-transcriptional mechanisms should also be considered such as actin-polymerization and recruitment of spines for encoding during spaced training (Kramar, Babayan et al. 2012), as well as protein phosphatases. Spaced learning trials inhibit PP1 activity via PKA phosphorylation of inhibitor 1 in mice leading to stronger activation of CREB and PKA phosphorylation of GluR1 (Genoux, Haditsch et al. 2002) and in *drosophila* spaced training induces repetitive waves of MAPK activation in a SHP2 dependent manner (Pagani, Oishi et al. 2009). Dopamine may also play a critical role the regulation of memory persistence after spaced training, something suggested by work in fruitflies (Placais, Trannoy et al. 2012), but to our knowledge not yet explored in mammals. Clearly, additional work is required to foster our understanding of the spacing effect on memory persistence – a phenomenon first described more than 130 years ago.

Cognitive enhancement: impact of Rolipram

We also observed that the PDE4 inhibitor Rolipram enhanced everyday memory at 24 h when given 45 min after encoding trials, but not when given 3 h after training. It is also worth noting that, in much of the literature examining the impact of Rolipram on memory, several previous studies have reported positive effects when the drug is applied prior to the learning trial, for example (Barad, Bourtchouladze et al. 1998, Zhang and O'Donnell 2000). This may eventually prove to be a 'valid' way for cognitive enhancers to be used in humans, but, from the perspective of scientific analysis, it

carries the ambiguity of being a procedure that confounds a potential impact on encoding (attention, memory acquisition) and an effect on consolidation (post-acquisition mechanisms). Our primary interest is the latter and, for that reason, we only tested post-trial injections of Rolipram. The effectiveness of the 45 min time-point is also of interest as various lines of research on long-term potentiation *in vitro* (at 32°C) suggest that synaptic tags, set at the time of memory encoding, have a life-time of around 1.5 h (Frey and Morris 1998a). Synaptic tags sequester plasticity-related proteins (PRPs). If Rolipram upregulates the availability of PRPs from the cAMP-PKA-CREB pathway, then an interval of 45 min is within the time window for effective capture by a synaptic tag. The effectiveness of Rolipram as a cognitive enhancer has been demonstrated in various tests of memory in naïve rats and mice, including contextual conditioning (Barad, Bourtchouladze et al. 1998), object recognition (Bourtchouladze, Lidge et al. 2003, Rutten, Prickaerts et al. 2006) and object location memory (Rutten, Van Donkelaar et al. 2009). Our study now extends this efficacy spectrum to everyday spatial memory in rats with extensive prior memorization experience. While Rolipram is not suitable for clinical development because of its emesis-inducing potential (ED50 (p.o.) = 0.03mg/kg (human nausea), 0.48mg/kg (rat pica); Percie du Sert, Holmes et al. 2012), next generation PDE4 inhibitors with a lesser emetic potential have been developed for CNS indications (Tully, Bourtchouladze et al. 2003, Burgin, Magnusson et al. 2010, Peters, Bletsch et al. 2014).

Theoretical importance of the impact of post-encoding environmental novelty

Novelty is often investigated in relation to reward, as in now classical experiments in which an animal's expectations of reward are violated and, for example, greater reward is made unexpectedly available (Schultz and Dickinson 2000). This is now known to activate dopaminergic signalling in the ventral tegmental area (VTA) that promotes effective learning (Montague and Sejnowski 1994). A different kind of novelty is unrelated to the task in hand, such as exposure to a novel stimulus or environment at some point before or after memory encoding of a separate task (Moncada and Viola 2007, Wang, Redondo et al. 2010). This novelty augments the retention of the unrelated but temporally proximate task in a dopamine-dependent manner with differential parts of the CaM Kinase pathway involved in tagging and plasticity-protein synthesis (Redondo and Morris 2011). The neuromodulatory mechanisms are still unclear, but an

intriguing new possibility is that novelty is mediated by activation of neurons in the locus coeruleus (LC)(Takeuchi, Duzskiewicz et al. 2016) that can co-release dopamine (Smith and Greene 2012, Kempadoo, Mosharov et al. 2016). This paradoxical dopaminergic modulation could trigger the synthesis of plasticity-related proteins linked to the stabilisation and thus retention of memory (Frey and Morris 1997, Redondo and Morris 2011). To date, several tests demonstrating enhanced memory with post-encoding novelty have used memory of a single stimulus or event (inhibitory avoidance, digging in a sandwell, object-place memory), but we sought to test whether novelty could also enhance the discriminability of two recent events, and the selective retention of the rewarded event relative to a non-rewarded event. The subsidiary study used the 2-sandwell encoding procedure with 3 massed rewards with only one sandwell rewarded. While memory of the cued sandwell location was at chance after 24 h, post-encoding novelty enhanced retention of the cued sandwell without enhancing memory of the non-cued trained location.

Evaluating cognitive enhancement

Much has been written about merits and possible drawbacks of cognitive enhancement, and about the strategies for achieving it ranging from behavioral approaches such as the ‘method-of-loci’ and retrieval practice (Roediger and Butler 2011), through to drugs that act at the time of encoding such as AMPAkinases (Lynch, Palmer et al. 2011) or during consolidation (Barco, Pittenger et al. 2003, Tully, Bourtchouladze et al. 2003). A final point relates to the suitability of animal models for testing putative enhancers. A critical factor is our present inability to ‘back-translate’ from human clinical data to animal models – largely because drugs that reliably enhance memory in humans have yet to be found. Minor differences in behavioural protocols in animal models may seem to be unimportant compared to the excitement of novel targets identified using the latest genetic techniques. However, we now assert that they have an important place in successfully identifying ‘hits’ and avoiding ‘false positives’. The present procedure can be contrasted with several other tasks of which one, context fear conditioning, is widely used by virtue of being such a rapid assay for the screening of genes and drug candidates. However, not only is it a slightly odd task compared to anything that normally happens to a person during the day, and thus of doubtful ‘construct validity’, it is generally tested in experimentally naive animals.

The 'lost-in-translation' problem that has led to too few drugs being developed for human use despite promising pre-clinical research may be exacerbated by a failure to recognize that a difference between animal and human trials with respect to cognition is not only the species but also the manner in which the testing is done. Prior knowledge is a key parameter. Recent work on schema learning in animals and humans (Tse, Langston et al. 2007, van Kesteren, Ruiter et al. 2012) has brought into sharp focus the issue of 'prior knowledge' in everyday memory function. The present task, while not schematic in nature, nonetheless relies on looking at single episode memory encoding in animals that have had extensive prior training. We now have an optimized version of the everyday memory task featuring: 1) sustained motivation and performance over months, 2) statistical advantages (within-animal design and less variability in probe test scores among animals), 3) objective scoring with good inter-observer agreement. Hopefully, this task could bridge the gap between rodent tests and human trials better than many in current use in preclinical trials. The gene expression analyses not only revealed for the first time in mammals that spaced encoding events induced more gene expression but the genetic resources obtained from RNA-seq would offer clues to potential targets for memory enhancement. Our approach, possibly supplemented by adding non-spatial recognition memory to the protocol, offers the prospect of revealing the effectiveness of new drugs that could translate effectively into successful Phase II trials for humans.

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CONFLICTS OF INTEREST

JL, DW, MP and TT are employees of Dart Neuroscience LLC which is involved in research and development associated with cognitive enhancement

FIGURE LEGENDS

FIGURE 1. *The 'event arena' apparatus and performance data.*

(A) The arena was 1.6 m x 1.6 m and made of clear plexiglass. It was open to extramaze cues in the laboratory. It had 4 start-boxes, with entry doors, and a 7 x 7 grid where floor panels can be placed. Some floor panels have circular holes into which sandwells could be placed. A ceiling mounted videocamera enabled observation of the animals. (B) The circular sandwells were constructed with semi-circular bowls with holes (left). The reward food (shown in brown) could either be in the bowl (accessible) or under the bowl (inaccessible) and were covered with sand mixed with masking flavor (middle and right). (C) Habituation protocol. On the first day [session (-8)], the arena location slots were filled with closed panels, and the rats habituated to the start-box (1 min) and to the arena (10 min). During the following habituation sessions [sessions (-7) to (-1)], one sandwell was open in the arena (in variable locations). Reward pellets were placed on top of the sandwells, but gradually buried deep in the sand to encourage digging. The rats were allowed to shuttle between the start-box and sandwells to retrieve more than one pellet. (D) Training schedules. Training session consists of ST (green shade) and CT that followed 60-90 min later. The location of the rewarded sample sandwell (filled circle) stayed in the CT. The 2-sandwell sample protocol is also shown as example. When stable performance was achieved, PT sessions were inserted once in every three

sessions. A probe trial (PT) is one in which the five reward pellets in all six sandwells are inaccessible (open circles). **(E)** Performance of the animals (n=23) that completed all phases of training. Note separate phases (colors), the sessions on which probe tests (PTs) were scheduled (gray), and the 5-session control phase (S58-S62) in which no sample trial (ST) was given on the third session (S60, pink shade). **(F)** Inter-observer correlation of probe test performance in a subset of 12 rats trained in the task. X-axis shows scores secured by MN in a semi-blind manner during the test itself, while Y-axis shows later blind scoring by an independent observer (RF). $R=0.996$, $p < 0.001$. Means ± 1 S.E.M. See also **Figure S1** and **Movie S1**.

FIGURE 2. *Delay-dependent forgetting and inter-observer correlation.*

(A) With the ST given as a single sandwell sample (green shading over event arena, left panels), we measured in the probe test, after various memory delays, the percent time digging at the correct sandwell (black arrow) and that at the other 5 sandwells. For the two-sandwell (2SW) procedure (right panels), digging was measured at all 6 sandwells, but now distinguished between the correct sandwell, the non-rewarded sandwell in the ST (gray arrow), and the untrained 4 sandwells. **(B)** For the 1SW procedure, the rats displayed delay-dependent forgetting over 24 h. **(C)** With the ST given as a 2SW sample (one of which was rewarded), delay-dependent forgetting over 24 h was also observed, but the plot now separates the cued (rewarded) and non-cued (non-rewarded) sandwells of the ST from the other 4 sandwells. The average variability in correct digging in B was 6.9% whereas was only 3.6% in C. ***: $p < 0.001$; ****: $p < 0.0001$. Means ± 1 S.E.M. Chance level = 16.7%. See also **Movie S2**.

FIGURE 3. *Impact of massed versus spaced access to reward during the sample trial.*

(A) Experimental design on probe tests with 30 s or 10 min intervals between the 3 reward pellets of the single sample trial. **(B)** Probe test performance at 2.4 h and 24 h as a function of massed vs. spaced training. Note sustained 24 h memory for spaced access. **(C)** Plot of Performance Index (PI) across the 3 separate entries to the arena during the sample trial (ST). The animals were at chance for the 1st pellet (indicating that the sandwell design was successful in preventing odor artifacts), but declined for pellets 2 and 3. The decline showed a trend towards a faster decline in errors within a

trial for massed access, despite long term retention over 24 h being more effective for spaced access. ***: $p < 0.001$; ****: $p < 0.0001$. Means \pm 1 S.E.M. See also **Movie S3**.

FIGURE 4. *Gene upregulation associated with task performance.*

(A) Timeline of massed or spaced sample training and tissue collection (left). Coronal rat brain to illustrate location of RSC and HPC (right). A tissue block of 4.0 mm AP was dissected out, but only bregma -3.80 mm is shown for simplicity. (B) (HPC) and (C) (RSC): RT-qPCR analysis from HPC tissue showed upregulation of IEGs (*Arc*, *c-Fos*, and *Npas4*) after either massed or spaced sample trials when compared to untrained controls, whereas two housekeeping (HPK) genes (*Actb* and *Tbp*) remained unchanged. *Gapdh* was used as the loading control and the data shown normalized to the control value of each gene. For the three IEGs, there was significantly greater upregulation in the spaced than massed reward condition. (D) (HPC) and (E) (RSC): Normalized FPKM values from RNA-Seq analysis of the same sets of IEGs and HPKs (including *Gapdh*). Note the similar trends as in B and C, respectively, for this method of gene-quantification. (F) RNA-Seq analysis of gene induction. Venn diagram showing the number of genes significantly induced ($p < 0.0001$ cut-off) after massed (left) and spaced (middle) training. For genes induced in both HPC and RSC in the massed (29 ‘massed’ genes) and the spaced conditions (54 ‘spaced’ genes), 2 genes were induced preferentially by massed training only, 27 genes by spaced training only, and 27 genes called for both massed and spaced (right). (G) Correlation of gene-induction levels for massed vs spaced conditions (HPC, 56 genes). The two genes with more expression in massed than spaced are highlighted in gray, and certain common IEGs of note amongst the 54 ‘spaced’ genes are named and highlighted in red. Dotted line indicates $y = x$ line. Green line is the linear regression of all 56 data points. The deviation from unity to 1.33 reflects overall stronger induction of genes after spaced training. (H) Same as G but for RSC tissue.

FIGURE 5. *The phosphodiesterase 4 inhibitor Rolipram enhances memory retention*

(A) Experimental design on probe tests 24 h later with i.p. injection of Rolipram (blue) at 45 min or 3 h after the single sample trial (ST) of single pellet retrieval. (B) Probe test performance at 24 h memory for Rolipram/vehicle injected groups. Rolipram injected 45 min after the ST successfully enhanced the memory at 24 h. **: $p < 0.01$; Means \pm 1 S.E.M. (See the main text for statistics.)

FIGURE 6. *Post-encoding environmental novelty enhances memory retention.*

(A) Experimental design on probe tests (PT) 24 min or 24 h after sample trial (ST) of either High reward or Low reward with or without 5 min of novelty exploration. (B) Probe test performance 24 min or 24 h after ST with or without post-encoding novelty, using 3-pellet reward (High reward). Black filled bars are cued-location, light-grey are non-cued trained; a grey surround to the bars denotes novelty. (C) Difference scores of relative searching at the Cued location vs. Non-cued trained location at each memory delay (High reward). Black and grey striping to denote subtraction. (D) Probe test performance 24 min or 24 h after ST with or without post-encoding novelty, using 1-pellet reward (Low reward). Note only transient memory at 24 min. (E) Difference scores of relative searching at the Cued location minus that to the Non-cued trained location at each memory delay (Low reward). **:p < 0.01; Means \pm 1 S.E.M. See also **Figure S2**.

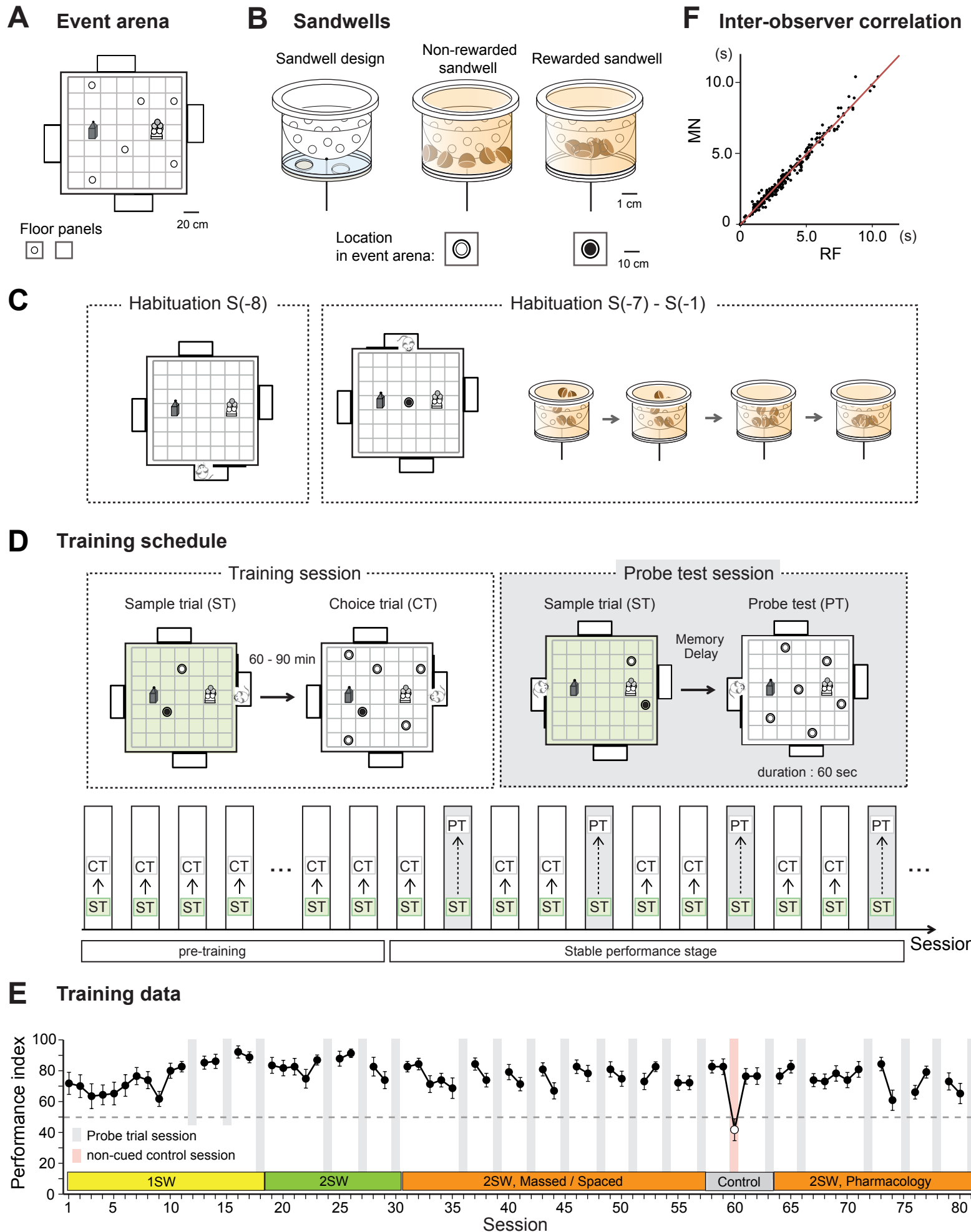
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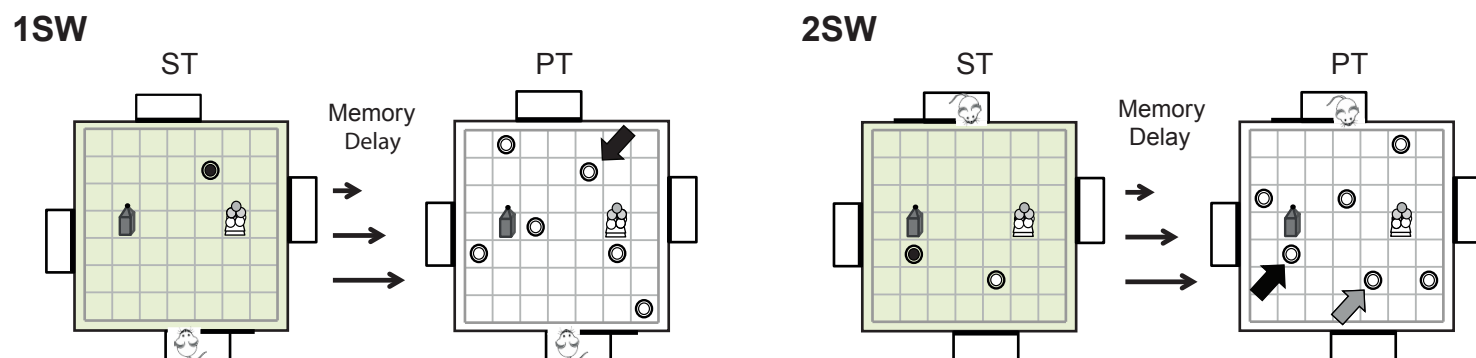
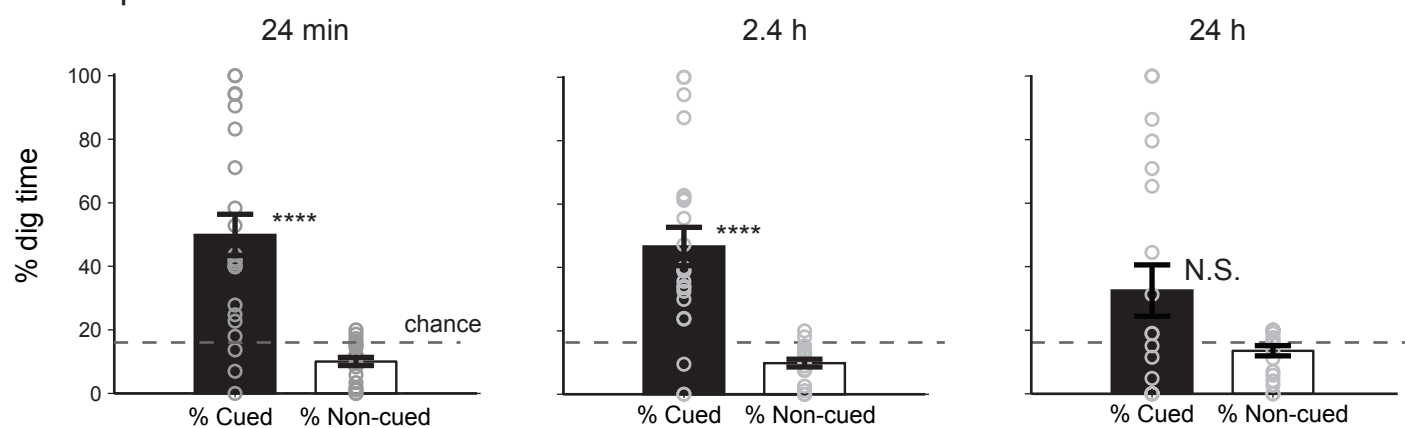
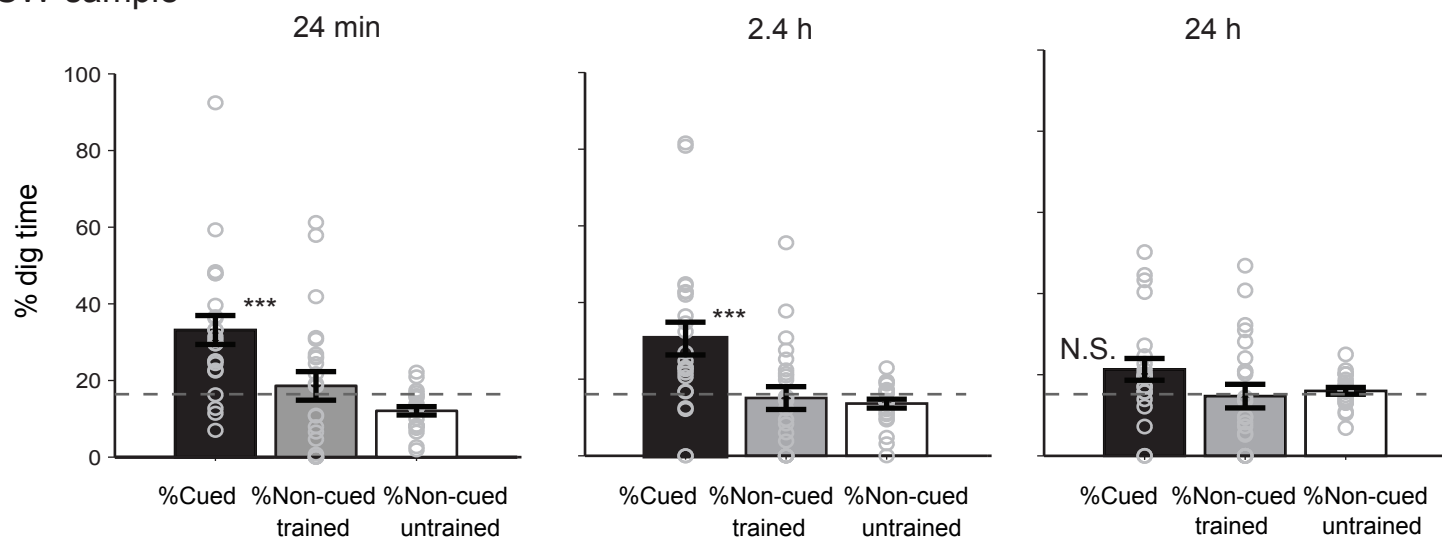
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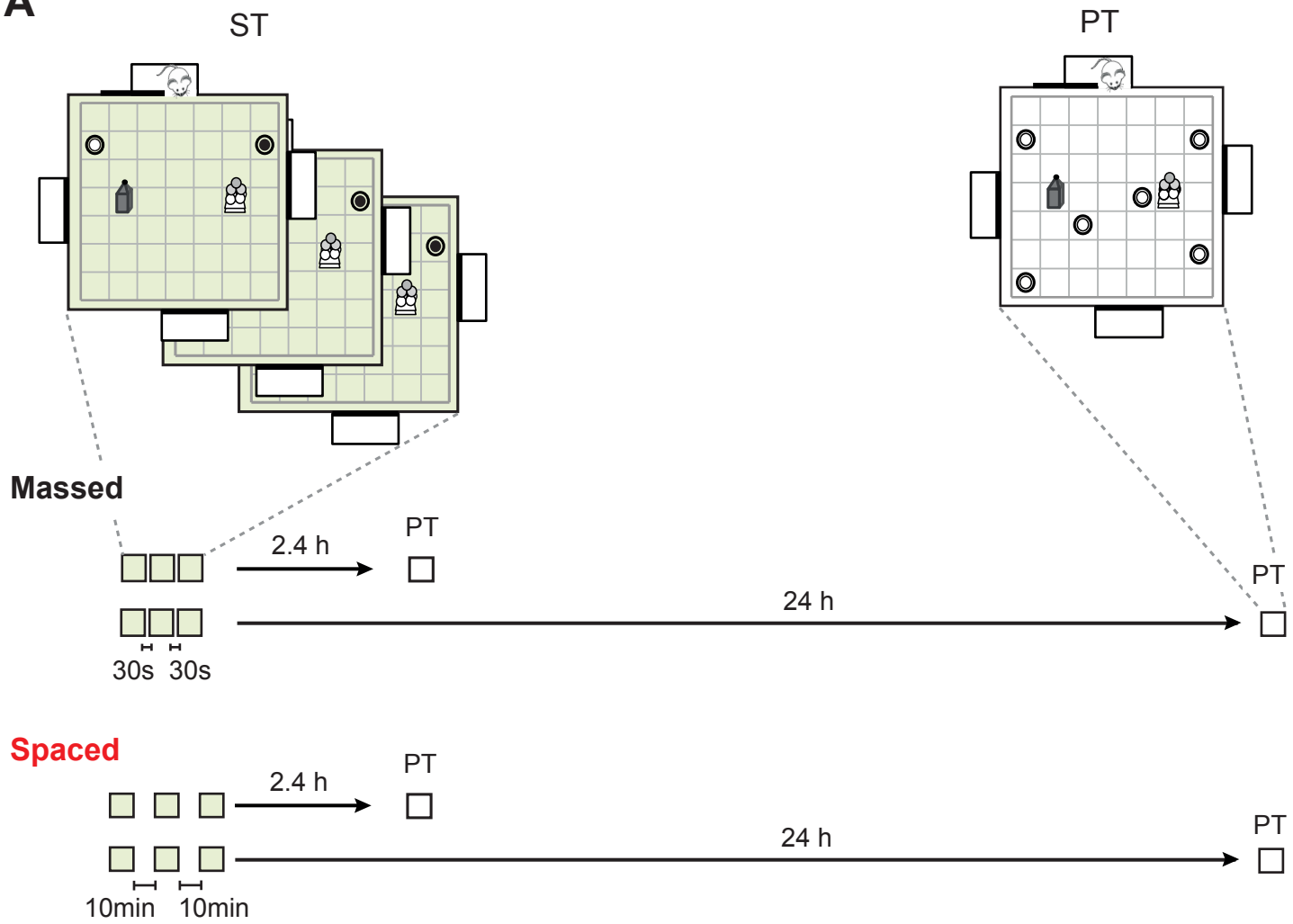
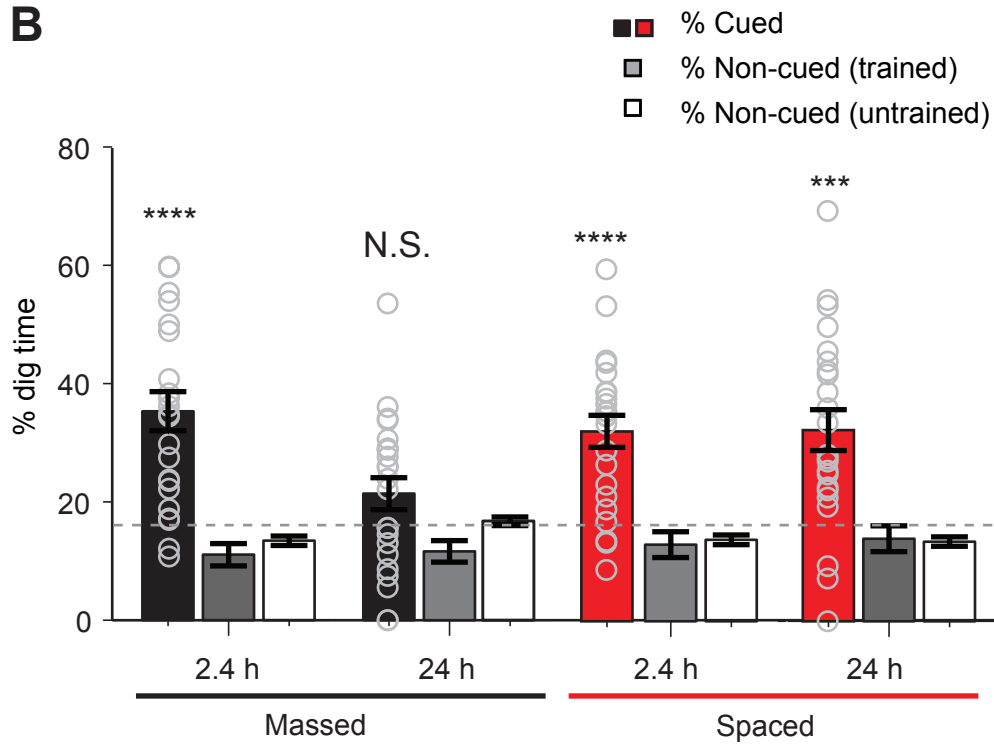
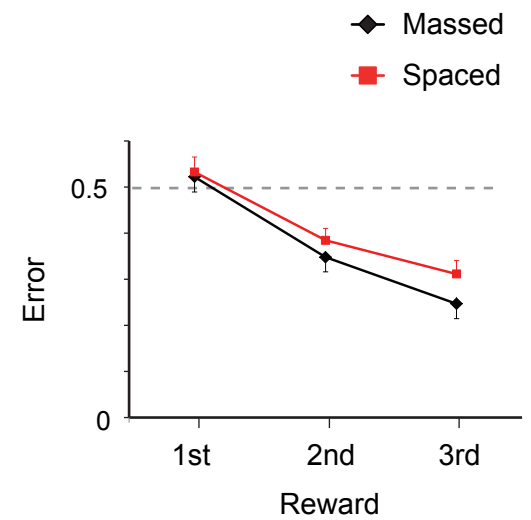
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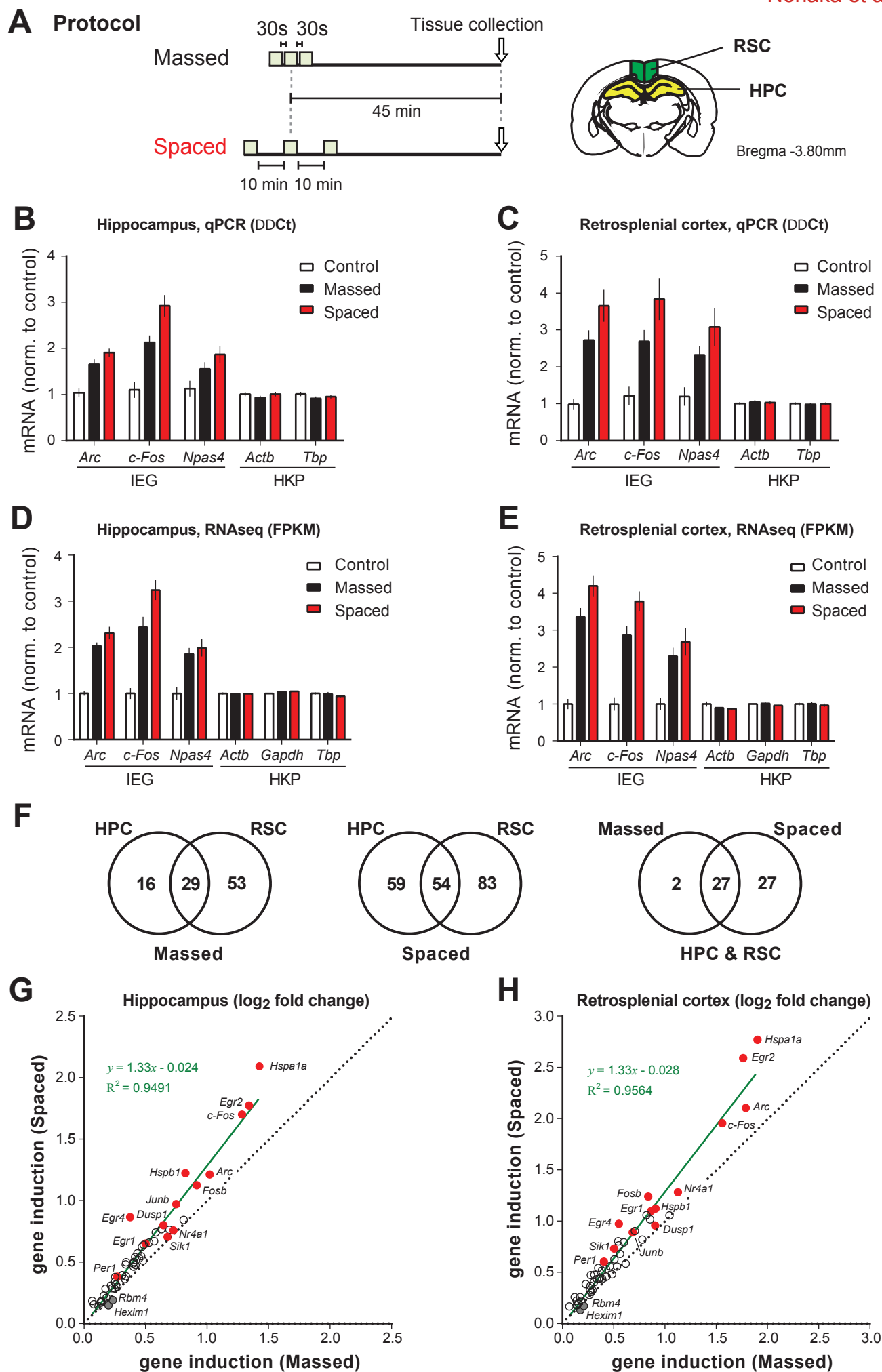
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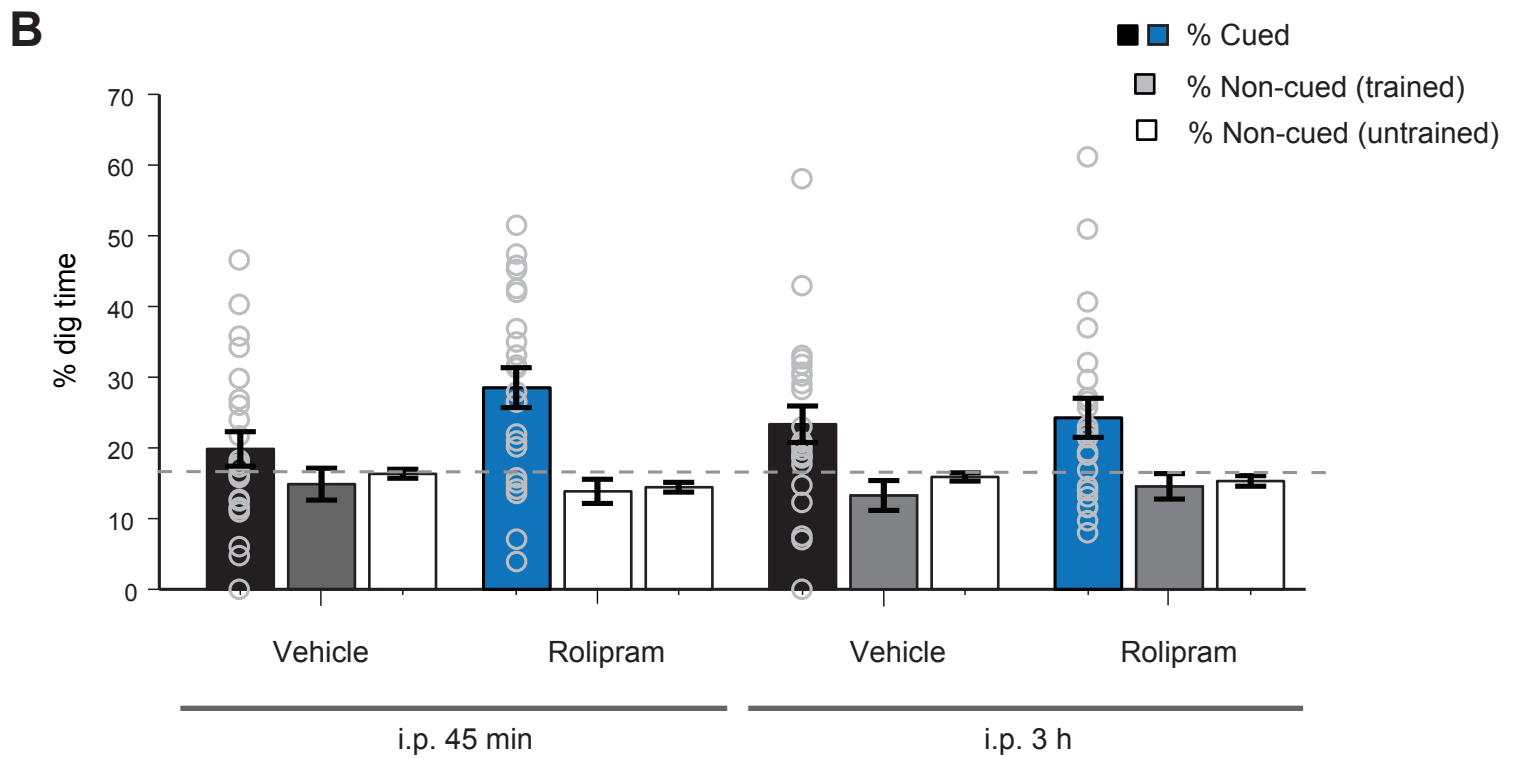
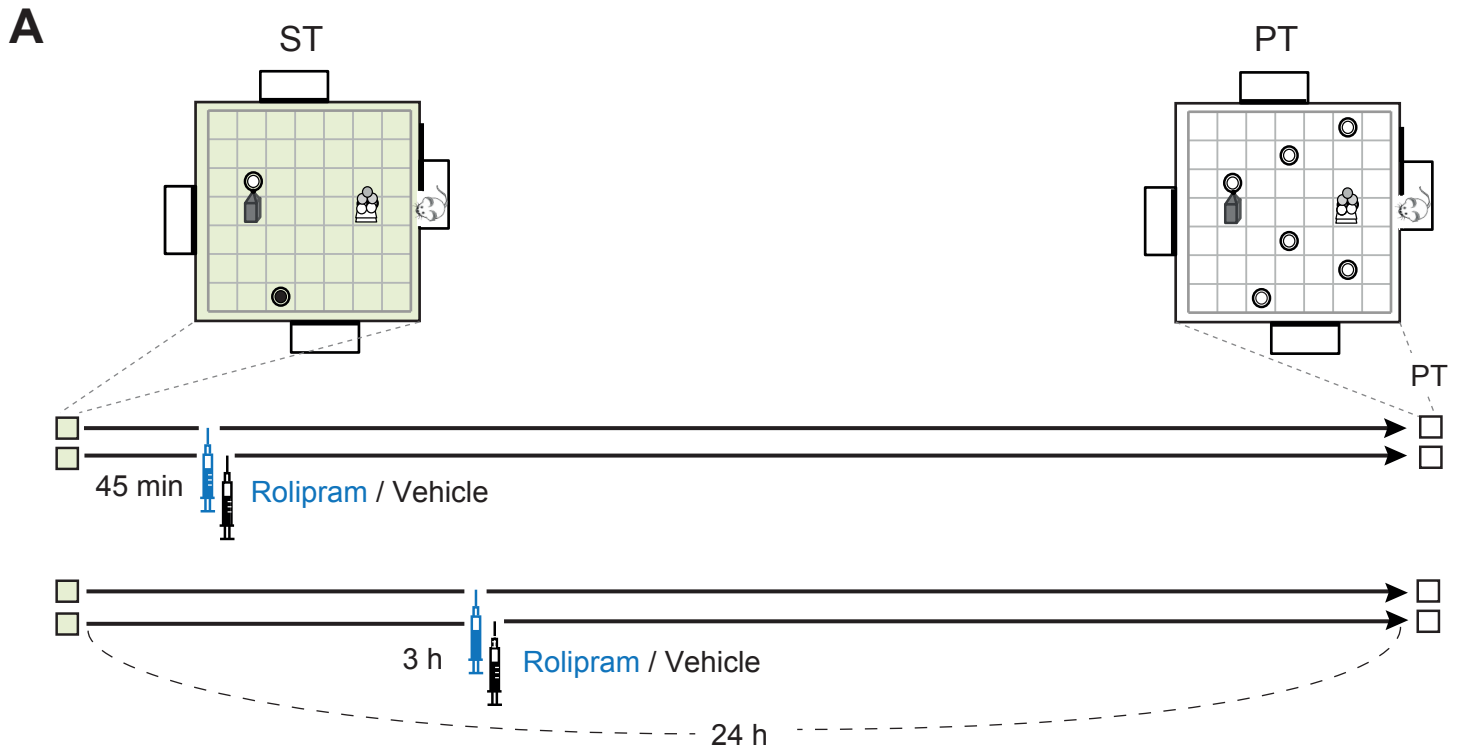
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A Probe test sessions after 1 or 2SW-sample encoding**B** 1SW-sample**C** 2SW-sample

A**B****C**





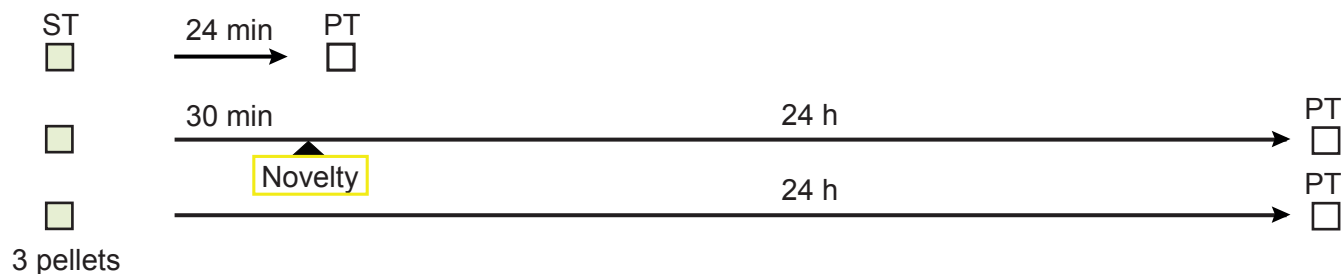
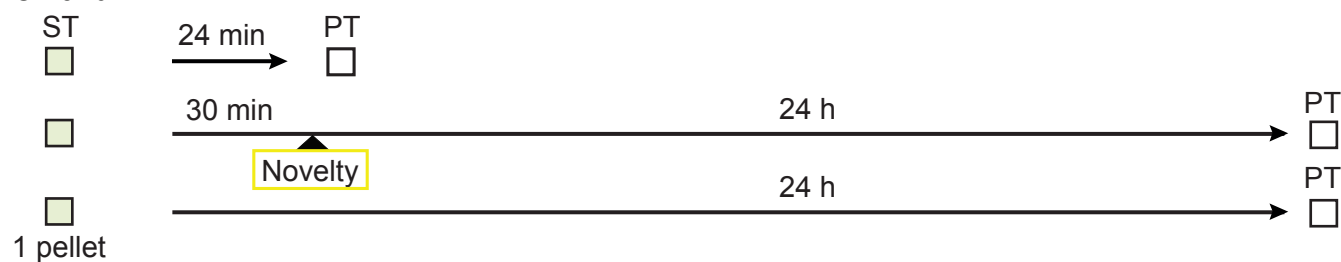
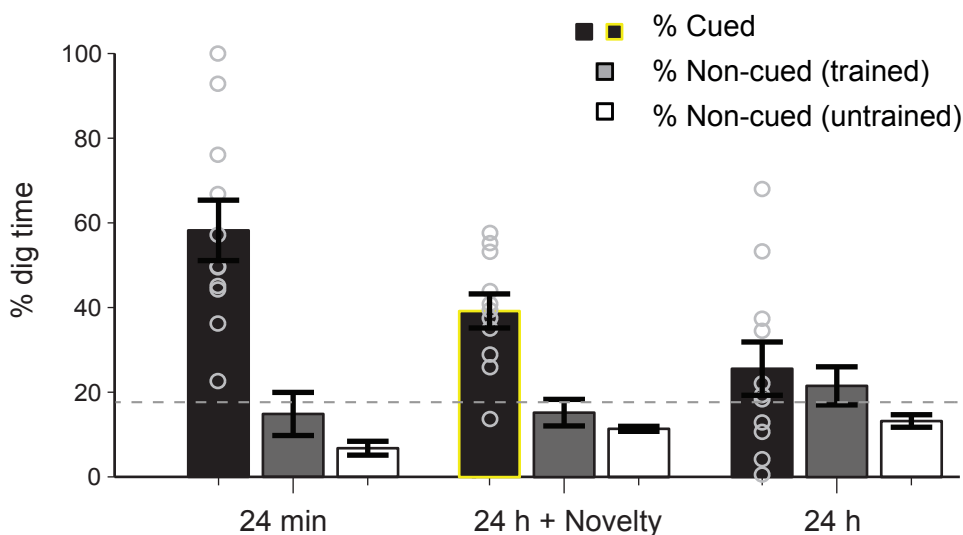
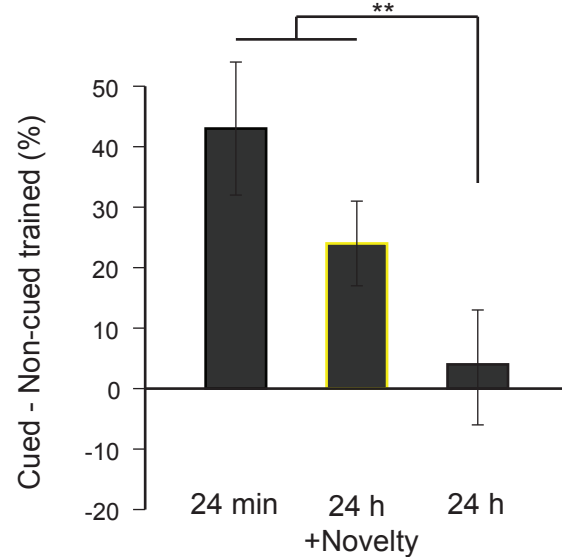
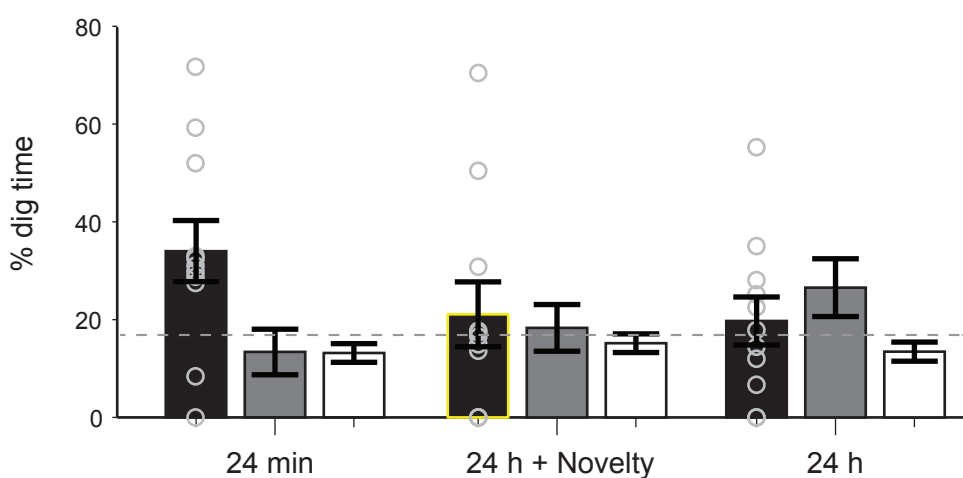
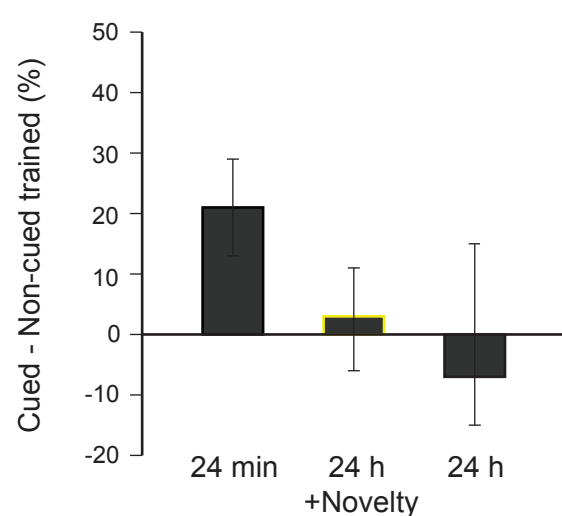
A**High reward****Low reward****B****High reward at encoding (3 pellets)****C****Difference score (High reward)****D****Low reward at encoding (1 pellet)****E****Difference score (Low reward)**

Figure S1 (related to Figure 1)

Training apparatus. (A) Photo of the event arena with a random 6 locations open onto which 6 sandwells were placed. (B) Photo of the distal spatial cues (the patterns on the walls and hanging cues) in the event arena room. (C) Photo of the sandwells. Rewarded sandwell (left) has accessible pellet hidden under the sand (not shown in the picture), and the non-rewarded sandwell (right) has inaccessible pellets below the porous plastic bowls in the sandwell. (D) Latency to first dig at the correct sandwell in the choice trials throughout the training sessions. Means \pm 1 S.E.M.

Figure S2 (related to Figure 6)

Plot of performance index (PI) across 52 sessions of training with the second cohort of animals used in the novelty study. After 11 sessions of training with a single sandwell during the sample trial, they were switched to a 2 sandwell sample trial, and this used during the later novelty phase. Note stability of above chance performance throughout the probe test sessions shown with grey shading. See also **Figure 1E**. Means \pm 1 S.E.M.

Table S1

Statistical information for RNAseq data.

Movie S1 (related to Figure 1)

Training sessions from ceiling camera view.

Two representative cases of the normal training session with 2-sandwell sample, 1-pellet retrieval paradigm for sample trials (ST). In STs, the rats choose the two sandwells randomly; in half the cases they first dig at the correct sandwell and never dig the other sandwell, while in other cases they visit and dig the correct sandwell as the 2nd choice. Both cases are shown in this video.

Movie S2 (related to Figure 2)

Probe trial sessions of 2-sandwell protocol from ceiling camera view.

(same as above) and the PT that followed 24 h after, indicating the memory decay over 24 h. In the PTs, the six sandwells are identical, but the cued location is indicated with red arrow and text.

Movie S3 (related to Figure 3)

Probe trial sessions of Massed vs Spaced from ceiling camera view.

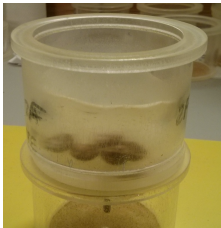
Two representative cases of Probe trial (PT) sessions for comparing massed and spaced access to reward. The first case shows the massed ST training (2-sandwell, 3 pellet retrieval, 30 s inter-reward interval (IRI)) and the PT that followed 24 h later, and the second case shows the spaced ST training (2-sandwell, 3 pellet retrieval, 10 min-IRI) and the PT that followed 24 h later.

A Event arena



C Sandwell

Rewarded sandwell (accessible pellets) Non-rewarded sandwell (inaccessible pellets)

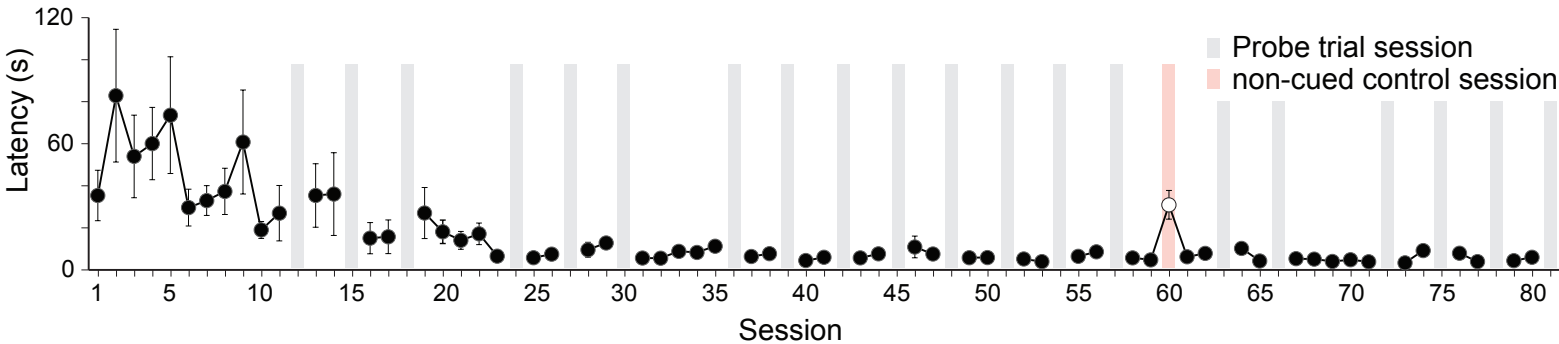


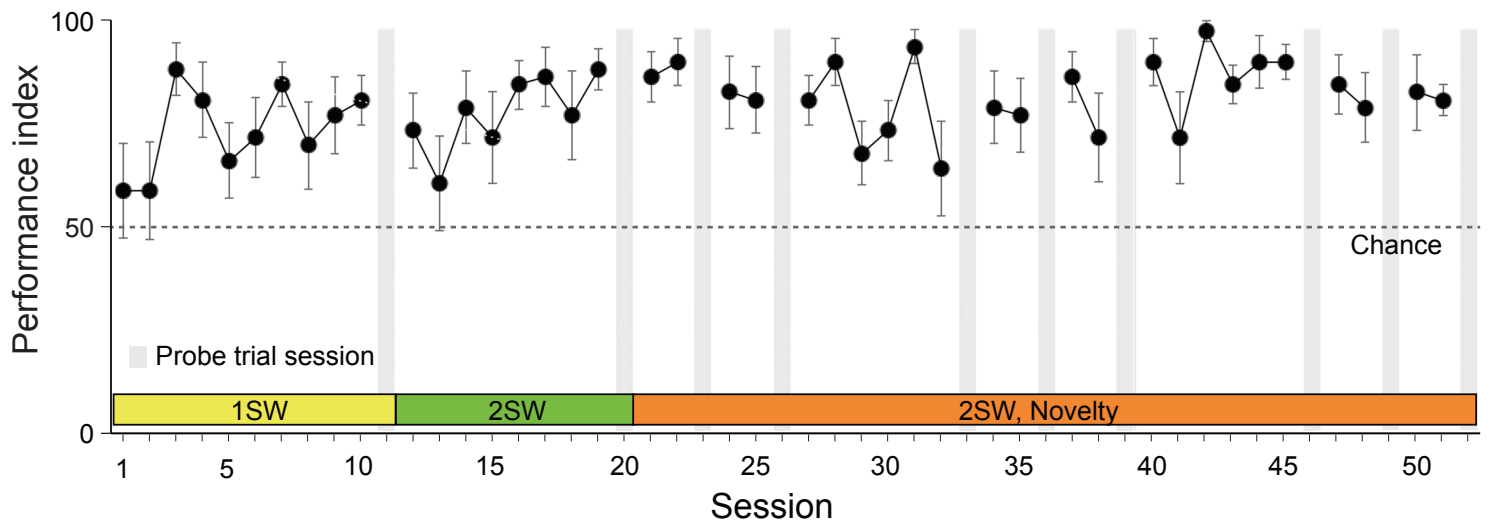
Intramaze cues

B Spatial cues



D





'Massed' vs 'Spaced' comparison in HPC:

Best-fit values \pm SE	
Slope	1.333 \pm 0.0397
Y-intercept	-0.02377 \pm 0.02279
X-intercept	0.01783
1/slope	0.7501
95% Confidence Intervals	
Slope	1.254 to 1.413
Y-intercept	-0.06947 to 0.02192
X-intercept	-0.01729 to 0.04973
Goodness of Fit	
R square	0.9543
Sy.x	0.09867
Is slope significantly non-zero?	
F	1127
DFn, DFd	1, 54
P value	<0.0001
Deviation from zero?	Significant
Equation	$Y = 1.333 \cdot X - 0.02377$

'Massed' vs 'Spaced' comparison in RSC:

Best-fit values \pm SE	
Slope	1.333 \pm 0.03644
Y-intercept	-0.02788 \pm 0.02664
X-intercept	0.02091
1/slope	0.7501
95% Confidence Intervals	
Slope	1.26 to 1.406
Y-intercept	-0.0813 to 0.02554
X-intercept	-0.02002 to 0.05853
Goodness of Fit	
R square	0.9612
Sy.x	0.1243
Is slope significantly non-zero?	
F	1338
DFn, DFd	1, 54
P value	<0.0001
Deviation from zero?	Significant
Equation	$Y = 1.333 \cdot X - 0.02788$